The interplay between hypoxia, endothelial and melanoma cells regulates vascularization and cell motility through endothelin-1 and vascular endothelial growth factor

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Reciprocal growth factor exchanges between endothelial and malignant cells within the hypoxic microenvironment determine tumor progression. However, the nature of these exchanges has not yet been fully explored. We studied the mutual regulation between endothelial cells (EC), melanoma cells and hypoxia that dictate tumor aggressiveness and angiogenic activity. Here, we investigated the presence of bidirectional autocrine/paracrine endothelin (ET)-1/ET receptor (ET1R)-signaling in melanoma cells, blood and lymphatic EC. In all these cells, hypoxia enhanced ET-1 expression, which in turn induced vascular endothelial growth factor (VEGF)-A and VEGF-C secretion, through the hypoxia-inducible growth factor (HIF)-1α and HIF-2α. Autocrine/paracrine exchanges of ET-1, VEGF-A and VEGF-C promoted tumor aggressiveness and morphological changes in blood and lymphatic EC. Furthermore, conditioned media from EC enhanced melanoma cell migration and vessel-like channel formation. This regulation was inhibited by ET1R blockade, by using the selective ET1R antagonist, or ET1R small interfering RNA (siRNA), and by VEGFR-2/3 antibodies, indicating that ET-1, VEGF-A/VEGF-C, produced by melanoma cells or EC mediated inter-regulation between these cells. Interestingly, HIF-1α/HIF-2α siRNA, impaired this reciprocal regulation, demonstrating the key role of these transcriptional factors in signaling exchanges. In melanoma xenografts, the ET1R antagonist reduced tumor growth and the number of blood and lymphatic vessels. These results reveal an interplay between melanoma cells and EC mediated by ET-1 and VEGF-A/C and coordinated by the hypoxic microenvironment through HIF-1α/HIF-2α transcriptional programs. Thus, targeting ET1R may improve melanoma treatment for tumor and EC, by inhibiting autocrine/paracrine signaling that sustains melanoma progression.

Introduction

Melanoma cells dynamically interact with stromal cells in a bidirectional manner through molecular signals that regulate the malignant phenotype. It is becoming increasingly apparent that metastatic dissemination is a complex process involving the interaction of tumor cells and endothelial cells (EC), in which signals from tumor cells and EC affect tumor growth and vascularization. Investigation into the molecular mechanisms that regulate tumor angiogenesis and lymphangiogenesis identified many host and tumor-derived angiogenic and lymphangiogenic factors, misregulation of which controls tumor vascularization (1,2). Several tumor-secreted angiogenic molecules and pathways responsible for stimulating EC activity are implicated in tumorigenesis. These include vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet-derived growth factor, angiopoietins, chemokines, integrins and endothelins (ETs) (3–9). However, the specific interactions of different stromal components, including EC, fibroblasts, soluble molecules and hypoxia in the tumor context, have only partially been clarified. Emerging evidence suggests that not only EC respond to tumor signals but also produce growth factors defined as “angiocrine factors” that enable tumor growth, motility and ultimately metastasis in a perfusion-independent manner (10).

ET-1 and its receptors have a relevant role in the growth and progression of several kinds of tumors (11). ET-1 has been reported to activate blood and lymphatic endothelium and promote melanoma progression via the activation of its receptor (ET1R) (11–13). The G-protein coupled ET1R is overexpressed in blood and lymphatic EC and melanoma cells (11,14–17). In these cells, activation of ET1R by its ligand ET-1 triggers cell proliferation, migration, invasiveness and induces the secretion of VEGF-A, VEGF-C and VEGF-D (18). Moreover, ET-1 may also transactivate the VEGF-C and VEGF-D receptor tyrosine kinase, the VEGF receptor 3 (VEGFR-3), enhancing melanoma cell mobility and invasiveness (18). Recently, ET-1 has emerged as a new potent mediator of angiogenesis and lymphangiogenesis in a mechanism that involves direct and VEGF-mediated actions (9,14,19–22). Therefore, the ET-1 axis, through its relationship with VEGF family members, plays a key role in tumor progression and metastasis.

Although ET-1 is produced by tumor and endothelial cells (11), how ET-1 regulates the interplay between melanoma and EC in the hypoxic microenvironment has not yet been investigated. In this study, we found that secreted ET-1, VEGF-A and VEGF-C from melanoma cells or from lymphatic and blood EC enhances migration and morphological changes in EC and melanoma cells, respectively. Given that the hypoxic tumor microenvironment plays a critical role in controlling several aspects of angiogenic programs, via the hypoxia-inducible growth factors (HIF)-1α and HIF-2α (23), we also addressed the role of hypoxia. Our results support a mechanism through which ET-1/ET1R regulates reciprocal communication between melanoma and EC and promotes melanoma invasive behavior, sustaining the development of a favorable microenvironment, recruiting vessels.

Materials and methods

Cell culture and small interfering RNA transfection experiments

Previously characterized lymphatic endothelial cells (LEC) (14) and human umbilical vein endothelial cells (HUVEC) (20) were grown in endothelial basal medium-2 (Lonza, Basel, Switzerland) containing 10% heat-inactivated fetal bovine serum supplemented with endothelial growth media-2 single quote (Lonza) grown in a humidified atmosphere at 37°C and 5% CO2. Human cutaneous melanoma cell lines were grown in RPMI 1640 containing 10% fetal calf serum. When the cells were exposed to hypoxia, oxygen deprivation was carried out in an incubator with 1% O2, 5% CO2 and 94% N2, and cells were grown for indicated times. Transfection was performed using Lipofectamine (Invitrogen) reagent following the manufacturer’s protocol. For silencing, small interfering RNA (siRNA) duplexes against human ET1R, HIF-1α or HIF-2α (ThermoFisher) were used. Non-target control siRNA (Dharmacon) was used as a control.
After 48h of incubation, cells were used for further experiments. Each knockdown experiment described herein was detected for specific reduced expression of ET<sub>R</sub>, HIF-1α or HIF-2α mRNA by quantitative real-time–PCR. For details, see the Supplementary Material, available at Carcinogenesis Online.

**Real-time–PCR**

Total RNA was isolated using the Trizol (Invitrogen) according to the manufacturer’s protocol. Five micrograms of total RNA were reverse transcribed using SuperScript®VILO™ complementary DNA synthesis kit (Invitrogen). Quantitative real-time–PCR was performed by using LightCycler rapid thermal cycler system (Roche Diagnostics, Indianapolis, IN) and LightCycler-FastStart DNA Master Plus SYBR Green mix (Roche Diagnostics). For details, see the Supplementary Material, available at Carcinogenesis Online.

**Enzyme-linked immunosorbent assay**

Subconfluent EC and melanoma cells were serum-starved for 24h and cultured for the indicated times. The conditioned medium was then collected, centrifuged and stored in aliquots at −20°C. The release of ET-1, VEGF-A and VEGF-C was measured on microtiter plates by an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. For details, see the Supplementary Material, available at Carcinogenesis Online.

**Wound-healing and Boyden chamber migration assays**

Cell migration was analyzed using wound-healing assay. Briefly, 15 × 10<sup>4</sup> cells were seeded in 6-well plates at a high density and were allowed to grow to confluence. Using a sterile 200 μl tip, a single scratch was made through the middle of each well. Photographs of wound closing were taken at the end of the experiment (24h) with Moticam Pro package (Motic Microscope, Wetzlar, Germany). Images were analyzed with ImageJ version 1.34s (http://rsb.info.nih.gov/ij/), and relative gap closure was measured as [1–(T<sub>24</sub>/T<sub>0</sub>)] using the same conditions to calculate percent relative gap closure. For Boyden chamber migration assay, 20 × 10<sup>4</sup> cells were plated on a transwell membrane filter inserts with 8 mm size polycarbonate membrane placed in a 24-well plate (BD Biosciences, Franklin Lakes, NJ), according to the manufacturer’s instructions. For details, see the Supplementary Methods, available at Carcinogenesis Online.

**Capillary-like structure formation assay**

The ability of endothelial and melanoma cells to form capillary-like structure formation has been assessed on cells cultured on matrigel (basal membrane extract; Trevigen, Gaithersburg, MD). Briefly, 30 × 10<sup>4</sup> HUVEC and LEC, or 25 × 10<sup>4</sup> 1007 cells, were plated. Images were analyzed with ImageJ version 1.34s (http://rsb.info.nih.gov/ij/) for determining the length of tubes and the number of intersections. For details, see the Supplementary Material, available at Carcinogenesis Online.

**Melanoma xenografts**

Female athymic (nu/nu) mice, 4–6 weeks of age (Charles River Laboratories, Milan, Italy), were handled in accordance with approved institutional guidelines under the control of the Italian Ministry of Health (DL 11692). Mice were injected subcutaneously on one flank with either 1.5 × 10<sup>6</sup> viable M10 or 1007 cells. After 7 days, when tumors reached ~0.2 to 0.3 cm in diameter, mice were randomized into groups (n = 10) to receive treatment intraperitoneally for 21 days with the selective non-peptide ET<sub>R</sub> antagonist A-192621 (10mg/kg/day; Abbott Laboratories, Abbott Park, IL), or 200 μl drug vehicle (0.25 N NaHCO<sub>3</sub>) for controls. All experiments were independently repeated three times, with a total of 20 mice in each experiment. All tumors for each group and for each experiment were harvested and quickly snap frozen from M10 and 1007 xenografts for western blotting and immunohistochemical analysis (Supplementary Material, available at Carcinogenesis Online).

**Statistical analysis**

Each experiment was repeated at least three times with comparable results, unless indicated otherwise. Statistical analysis was carried out by using the Student’s t-test or one-way analysis of variance as appropriate. All statistical tests were two-sided and were performed using PRISM software (version 5.0, GraphPad Prism, San Diego, CA). P-values were significant at P < 0.05.

**Results**

**ET-1, VEGF-A and VEGF-C production is controlled by hypoxia in melanoma cells, blood and LEC**

ET-1, VEGF-A and VEGF-C represent the principal mediators of angiogenesis and lymphangiogenesis, and recent data argued for a more relevant role of these factors in controlling melanoma invasive behavior (17). Therefore, we investigated the regulation of their expression in primary (1007) and metastatic (M10, M14 and SKMel28) melanoma cell lines, and in blood (HUVEC) and LEC, under normoxic and hypoxic conditions. As previously reported for LEC and HUVEC (24), we showed for the first time that melanoma cells, expressing high levels of ET<sub>R</sub> (16), express and release ET-1 (25–50ng/ml/10<sup>6</sup> cells; Figure 1A and B). Hypoxia induced a time-dependent increase in ET-1 mRNA and protein expression, concomitantly with an increase in VEGF-A and VEGF-C secretion (Figure 1 and Supplementary Figure 1A, available at Carcinogenesis Online).

The use of the selective antagonist of ET<sub>R</sub>, BQ788 (15), as well as of siRNA against ET<sub>R</sub>, significantly (P < 0.001) reduced the basal and the hypoxia-induced ET-1, VEGF-A and VEGF-C production in melanoma cells, LEC and HUVEC (Figure 1B and Supplementary Figure 1B, available at Carcinogenesis Online). These results demonstrated that melanoma cells released ET-1 under normoxic conditions and to a greater extent under hypoxic conditions, suggesting that ET-1 may participate in hypoxia-induced VEGF-A and VEGF-C expression through the autocrine activation of ET<sub>R</sub> in both melanoma and EC.

**HIF-1α and HIF-2α mediate hypoxia-induced ET-1, VEGF-A and VEGF-C expression in melanoma and endothelial cells**

ET-1 shares similar transcriptional properties with VEGF having the hypoxic responsive elements capable to bind HIF-1α on their promoters under both hypoxic and non-hypoxic conditions (25). Therefore, we evaluated whether HIF-1α and HIF-2α were involved in the hypoxia-dependent induction of ET-1, VEGF-A and VEGF-C, in melanoma cells, LEC and HUVEC. We found that the silencing of HIF-1α and HIF-2α resulted in a significant reduction in ET-1, VEGF-A and VEGF-C mRNA and protein expression under hypoxic conditions (Figure 2 and Supplementary Figure 2A, available at Carcinogenesis Online). Furthermore, under these conditions, the capacity of ET-1 to increase VEGF-A and VEGF-C mRNA and protein expression in melanoma and EC was reduced (Supplementary Figure 2B, available at Carcinogenesis Online). These data suggested the intriguing hypothesis that hypoxia induces production of ET-1 that, in turn, may sustain HIF-1α/HIF-2α-mediated VEGF-A and VEGF-C expression in melanoma cells and EC.

**HIF-1α- and HIF-2α-mediated autocrine production of ET-1 controls melanoma cell migration and vasculogenic mimicry**

We previously demonstrated that, in response to ET-1, melanoma cells enhance their capacity to migrate and form vasculogenic-like network in vitro, a process known as ‘vasculogenic mimicry’ through which highly aggressive tumor cells acquire endothelial features (18,26). To evaluate the functional role of melanoma-secreted factors and the role of ET-1/ET<sub>R</sub> axis on melanoma aggressive behavior, we measured the capacity of ET<sub>R</sub>-silenced cells to migrate and to engage morphological changes. In melanoma cells, wound-healing (Figure 3A, upper panel) and chemotaxis assays (Figure 3A, lower panel) demonstrated that ET<sub>R</sub>-silenced cells showed migration to a much less extent compared with the control, suggesting that autocrine production of ET-1 is required for melanoma cell migration. Under hypoxic conditions, cells spread and migrated to a greater extent, as evaluated by quantification analysis (Figure 3A). In these conditions, siHIF-1α/2α as well as siET<sub>R</sub>-transfected melanoma cells showed a reduced capacity to migrate (Figure 3A). Moreover, in the presence of rhVEGFR-2 and rhVEGFR-3, neutralizing antibodies for VEGF-A and VEGF-C, melanoma cells showed a reduced migration capacity (Figure 3A), demonstrating that ET-1, VEGF-A and VEGF-C are responsible for the induction of cell migration under hypoxia. In addition, these results demonstrated that HIF-1α and HIF-2α are the transcriptional factors involved in hypoxia and ET-1-induced VEGF-A/C signaling. In a three-dimensional culture assay, where it is possible to measure the capacity of cells to organize in tube-like structures, serum-starved melanoma cells elongated their processes and formed few clusters connected to a network of structures similar to tubules (Figure 3B). On the contrary, ET<sub>R</sub>-silenced melanoma cells slightly elongated their process and formed...
small tubules aggregates (Figure 3B). Quantification analysis demonstrated that the length of tubes and the number of their intersections decreased significantly ($P < 0.01$) compared with the control (Figure 3B). When cells were exposed to hypoxia, their capacity to form vascular-like structures significantly ($P < 0.01$) increased by ~3-fold compared with the cells cultured under normoxic conditions, whereas cells transfected with siRNA targeting HIF-1α/HIF-2α were unable to develop elongated structures and formed few interconnections. In the siET$_R$-transfected cells, as well as in cells pretreated with rhVEGFR-2 and rhVEGFR-3, vasculogenic mimicry of
melanoma cells was significantly impaired (Figure 3B). Our results indicate that hypoxia might induce autocrine production of ET-1, VEGF-A and VEGF-C that are responsible for the induction of aggressive behavior in melanoma cells.

**ET-1 and VEGF released by melanoma cells under hypoxia conditions influence migration and capillary-like structure formation in blood and LEC**

To determine how melanoma-secreted factors can regulate EC behavior, we monitored EC’s ability to migrate and form tube-like structures in response to conditioned media (CM) from 1007 cells. To minimize the effects of exogenous growth factors, HUVEC and LEC were plated in serum-free medium. Under these conditions, EC showed a low capacity to migrate, covered 20% of the scratched area and formed small tight clusters with few sprouting and elongation in matrigel (Figure 4A and B). In the presence of melanoma CM, EC increased their migration reaching 70% of gap closure and displayed increased network formation (4-fold induction), demonstrating that melanoma secreted factors regulated the formation of tube-like structures in EC. We further evaluated ET-1, VEGF-A and VEGF-C contribution to EC activation induced by melanoma CM. Melanoma CM-induced migration (Figure 4A) and tube-like structure formation (Figure 4B) of EC, pretreated with BQ788 or with neutralizing antibodies against VEGFR-2 and VEGFR-3, were inhibited. Moreover, CM from melanoma cells silenced for HIF-1α and HIF-2α was unable to induce cell migration and cord formation in LEC and HUVEC (Figure 4). Notably, CM from 1007 cells exposed to hypoxia further increased EC migration and cord formation, whereas pretreatment of EC with BQ788 significantly impairs these actions (Figure 4). These results demonstrated that melanoma cells are able to release ET-1, VEGF-A and VEGF-C and induce angiogenic properties in both blood and lymphatic EC and that hypoxia increases this paracrine regulation.

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**Fig. 3.** HIF-1α- and HIF-2α-mediated autocrine production of ET-1 controls melanoma cell migration and vasculogenic mimicry *in vitro*. 1007 cells were seeded in serum-free media and transfected with SCR siRNA or siRNA against ET₄R or HIF-1α and HIF-2α, or treated with rhVEGFR-2 and rhVEGFR-3. Cells were then cultured for 24h under normoxic or hypoxic conditions. (A) Cell migration was analyzed by wound-healing (upper panel) and chemotaxis (lower panel) assays. Representative images of cells allowed to close the wound and to migrate in a transwell invasion chamber are shown. The quantification of wound closure and of transwell migration was measured and expressed as percentage of gap closure and percentage of migrated cells. (B) Formation of vascular-like structures was examined and quantification analysis was performed by measuring tube length and number of intersections. A and B, data are means ± SD from 10 independent experiments each performed in duplicate. *P < 0.01 (versus normoxia SCR); **P < 0.001 (versus hypoxia SCR).
Hypoxia-induced endothelial cell-released ET-1, VEGF-A and VEGF-C promote melanoma cell migration and vasculogenic mimicry

To determine the reciprocal signaling regulation between melanoma and endothelial cells, we analyzed whether EC-released factors may influence melanoma cell behavior. To this end, we compared melanoma cell migration and vasculogenic mimicry in the presence or absence of CM from LEC or HUVEC. Melanoma cells exposed to EC CM consistently filled the wound (Figure 5A) reaching 60% of gap closure and formed an extensive network of tube-like structures (Figure 5B). These effects were inhibited when melanoma cells were treated with BQ788, demonstrating that EC-secreted ET-1 signals are implicated in the induction of melanoma morphological changes through ET\textsubscript{B}R. Similarly, when melanoma cells were pretreated with rhVEGFR-2 and rhVEGFR-3, cell migration and vasculogenic mimicry were strongly inhibited (Figure 5), suggesting that ET-1, VEGF-A and VEGF-C influenced melanoma cell motility and aggressiveness. When melanoma cells were cultured in the presence of CM from HIF-1\textalpha/HIF-2\textalpha–silenced EC, their migration and vasculogenic capacity were drastically reduced, thus supporting HIF-1\textalpha and HIF-2\textalpha key role in controlling EC paracrine functions. To further investigate the role of the hypoxic microenvironment in this process, we stimulated 1007 cells with CM from EC exposed to hypoxia (Figure 5B). These effects were inhibited when melanoma cells were treated with BQ788, demonstrating that EC-secreted ET-1 signals are implicated in the induction of melanoma morphological changes through ET\textsubscript{B}R. Similarly, when melanoma cells were pretreated with rhVEGFR-2 and rhVEGFR-3, cell migration and vasculogenic mimicry were strongly inhibited (Figure 5), suggesting that ET-1, VEGF-A and VEGF-C influenced melanoma cell motility and aggressiveness. When melanoma cells were cultured in the presence of CM from HIF-1\textalpha/HIF-2\textalpha–silenced EC, their migration and vasculogenic capacity were drastically reduced, thus supporting HIF-1\textalpha and HIF-2\textalpha key role in controlling EC paracrine functions.

Blockade of ET\textsubscript{B}R activity affects tumor growth, angiogenesis and lymphangiogenesis in vivo

1007 or M10 cells were inoculated subcutaneously in nude mice and when the tumor was palpable (~0.3 cm in diameter), the mice were treated with A-192621, a selective orally active ET\textsubscript{B}R antagonist (13,27) (10 mg/kg/day). Continuous treatment for 21 days produced a 52% and 41% reduction of tumor growth on day 30 after tumor injection in 1007 and M10 xenografts, respectively (Figure 6A), concomitantly with a reduction of VEGF-A and VEGF-C and the lymphatic marker lymphatic vessel endothelial hyaluronan receptor (LYVE-1) (14) expression, compared with the control (Figure 6B). The immunohistochemical results revealed a significant and homogenous decrease of CD31 and LYVE-1 staining in tumors from treated mice, which paralleled the ability of A-192621 to reduce tumor growth. The quantification analysis showed that treatment with A-192621 significantly (P < 0.001) inhibited blood (10 ± 3) and lymphatic (2.27 ± 1.5) microvascular density compared with untreated xenografts (41 ± 4 and 9.75 ± 1.5, respectively; Figure 6C and D). These data suggest that factors regulated by ET\textsubscript{B}R, including ET-1 and VEGF-A and VEGF-C, may sustain tumor growth by recruiting vessels, and moreover, that ET\textsubscript{B}R...
blockade may impair the reciprocal influence between endothelial and tumor cells.

Discussion

Metastatic progression is a complicated interplay between signaling molecules, tumor cells and endothelial cells that are differentially modified by the tumor microenvironment (28). This study has related the reciprocal ability of melanoma, blood and LEC to modulate cell migration, differentiation and tube formation to their ability in secreting ET-1, VEGF-A and VEGF-C. This inter-relation was enhanced by hypoxia, that by increasing ET-1, VEGF-A and VEGF-C expression on endothelial and melanoma cells, sustained an autocrine loop that amplified the invasive signaling in these cells. More importantly, we discovered the existence of a HIF-1α/2α-mediated mechanism that mediates a hypoxic-dependent ET-1 autocrine loop in melanoma cells that was amplified by hypoxia via HIF-1α and HIF-2α. Here, we found that autocrine production of ET-1 was involved in hypoxia-increased VEGF-C and VEGF-A, demonstrating that melanoma cells may represent a source of ET-1 production and that hypoxia and ET-1 may activate an autocrine positive loop that contributes to the increase in VEGF family member expression, via HIF-1α and HIF-2α.

Fig. 5. Hypoxia-induced endothelial cell-released ET-1, VEGF-A and VEGF-C promote melanoma cell migration and vasculogenic mimicry. 1007 cells were seeded on serum-free media in the absence (C) or presence of BQ788, or rhVEGFR-2 and rhVEGFR-3, and treated with CM from LEC or HUVEC cells untransfected or transfected with siRNA against HIF-1α and HIF-2α, or exposed to hypoxia (H). (A) Cell migration was monitored and the relative rate of wound closure was measured and expressed as percentage of gap closure. (B) Formation of vascular-like structures was examined and quantification analysis was performed by measuring tubule length and number of intersections. A and B, data are means ± SD from 10 independent experiments each performed in duplicate. *P < 0.01 (versus C); **P < 0.001 (versus CM from EC); ***P < 0.001 (versus H CM).
growth factors (31). These LEC-derived factors might also function as hemangiogenic factors, which may further enhance tumor-induced angiogenesis. In this regard, it has been demonstrated that blood EC produce lymphangiogenic factors, such as VEGF-C, to facilitate tumor-induced lymphangiogenesis other than stimulate tumor cell growth (32,33). The bilateral interplay of tumor, blood and lymphatic compartments are all beneficial for tumor growth (34). Our data support the notion that endothelial and melanoma cells might regulate each other by the secretion of common growth factors such as ET-1, VEGF-A and VEGF-C, resulting in increased neovascularisation and cell motility. Indeed, impairing the ET-1- and VEGF-mediated signaling from EC to tumor cells results into inhibition of cell motility and acquisition of aggressive phenotype.

Melanoma is characterized by the propensity of tumor cells to metastasize via blood and lymphatic blood vessels (35), indicating a strong and specific relationship between melanoma and endothelial cells in the tumor context. Moreover, emerging evidence supports the hypothesis that growth factors released by tumor cells may modify the microenvironment within the vascular system, even before arrival of tumor cells, providing a favorable niche for the survival and growth of arriving tumor cells (36). Providing that ET-1 together with VEGF-A/C are actively secreted by EC and play crucial roles in melanoma cell migration and invasion, our data suggest that ET-1 may represent one of such soluble factors that mediates the communication between tumor and endothelial cells favoring a permissive environment for growth and invasion. At mechanistic level, we found that silencing of HIF-1α and HIF-2α completely inhibits melanoma capillary-like structure formation indicating that the EC’s influence on melanoma cells is controlled by HIF-transcriptional mechanism. In agreement with our results, a recent report demonstrated that chondrosarcoma xenografts, knockdown of ET-1 significantly reduced VEGF expression and also abolished chondrosarcoma CM-mediated angiogenesis. This mechanism is regulated by ET-1-induced HIF-1α expression and stability, showing that HIF-1α controls ET-1-induced angiogenesis in human chondrosarcomas (37). Similarly, a recent study reported that HIFs mediated complex and bidirectional paracrine signaling between breast cancer cells and mesenchymal stem cells (38,39), further supporting the role of HIF1α-target genes on controlling tumor/stromal cell communications.

It is now recognized that tumors can acquire a blood supply system through alternative means including recruitment of endothelial progenitor cells and induction of tube-like structure formation by tumor cells (26,40). Although gene expression profiling of melanoma cell lines has identified ETaR as one of the genes associated with a more aggressive phenotype, characterized by the capacity to organize vasculogenic mimicry (16), the mechanism by which EC could modulate this alternative angiogenic network through ET-1/ETaR has not yet been fully investigated. In this study, we demonstrated that hypoxia-stimulated ET-1, through HIF-1α, engages melanoma cells in tubular network formation. Recent studies reported that aggressive cells capable of vasculogenic mimicry expressed genes of stemness and may contribute to perivascular niche morphogenesis and tumorigenesis (41,42). Interestingly, ET-1 expression has been associated with features expressed by a highly tumorigenic subpopulation of cancer stem cells (41–44). In this context, our findings demonstrating the capacity of ET-1/ETaR axis to regulate melanoma as well as tumor stroma plasticity, by enhancing vasculogenic ability and acquisition of stem cell traits, suggest that ETaR pathway could promote the survival and maintenance of stemness in a favorable metastatic niche.

Hypoxia is the major pathophysiological condition regulating angiogenesis and capillary morphogenesis (45). Indeed, exposing these cells to CM from EC or melanoma cells cultured under hypoxia resulted in enhancing their ability to migrate or to engage in vasculogenic mimicry. In view of the reported cooperation between ET-1 and hypoxia in the angiogenic process and tumor aggressive behavior (11), our findings demonstrate that hypoxia pathway progresses also through ET-1 and VEGF-C/A to increase EC and melanoma cord formation. Therefore, it is possible to limit tumor microcirculation targeting ETaR, expressed on endothelial and non-endothelial cells, for ET-1-mediated direct effects and VEGF-mediated effects.
These results shed new light on the ability of ET-1/ET\(_4\)R axis to affect the interplay between lymphatic, blood, tumor cells and hypoxia sustaining the development of melanoma growth in a favorable microenvironment. Indeed, our present in vivo study demonstrated that mice bearing melanoma xenograft showed a slowed tumor growth rate in response to 21 days continuous treatment with ET\(_4\)R antagonist, concomitantly with a reduction of both blood and lymphatic microvascular density, indicating that ET\(_4\)R blockade can hamper the bidirectional communications between LEC, blood EC and melanoma cells mediated by ET-1, VEGF-A and VEGF-C, that are all beneficial to create a permissive environment for melanoma growth. Regarding the therapeutic implications of these findings, we should consider that although promising preclinical data have been obtained by using small-molecule ET\(_4\)R antagonists, they have not yet been translated into the clinical setting. To decipher why the clinical results have been disappointed, it could be argued that pharmacological ET\(_4\)R blockade could tilt the balance toward increased ET\(_4\)R signaling in the tumor microenvironment. Indeed, besides pro-angiogenic signaling, ET\(_4\)R signals may also impede antimelanoma immunity by preventing the maturation and function of dendritic cells, which are pivotal for the initiation of T-cell-mediated immune responses and the homing of T cells to tumors (46). This could partly explain the failure of specific ET\(_4\)R antagonists to produce significant clinical results in tumors in which the inhibition of an antitumor immune response might affect overall survival (11,46). In view of this, preclinical data obtained with the ET\(_4\)R antagonist indicate that this class of drugs, or the non-selective ET\(_4\)R antagonist, could be a promising therapeutic option for tumor treatment for its capacity to target cancer cells as well as tumor-infiltrating immune cells, lymphatic and blood EC.

The improved knowledge of ET-1 autocrine and paracrine signaling to melanoma progression through a network of cellular pathways and interactions within the hypoxic microenvironment will allow the exploration of novel therapeutic strategies with agents capable to blockade ET\(_4\)R.

Supplementary material

Supplementary Material, Methods and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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